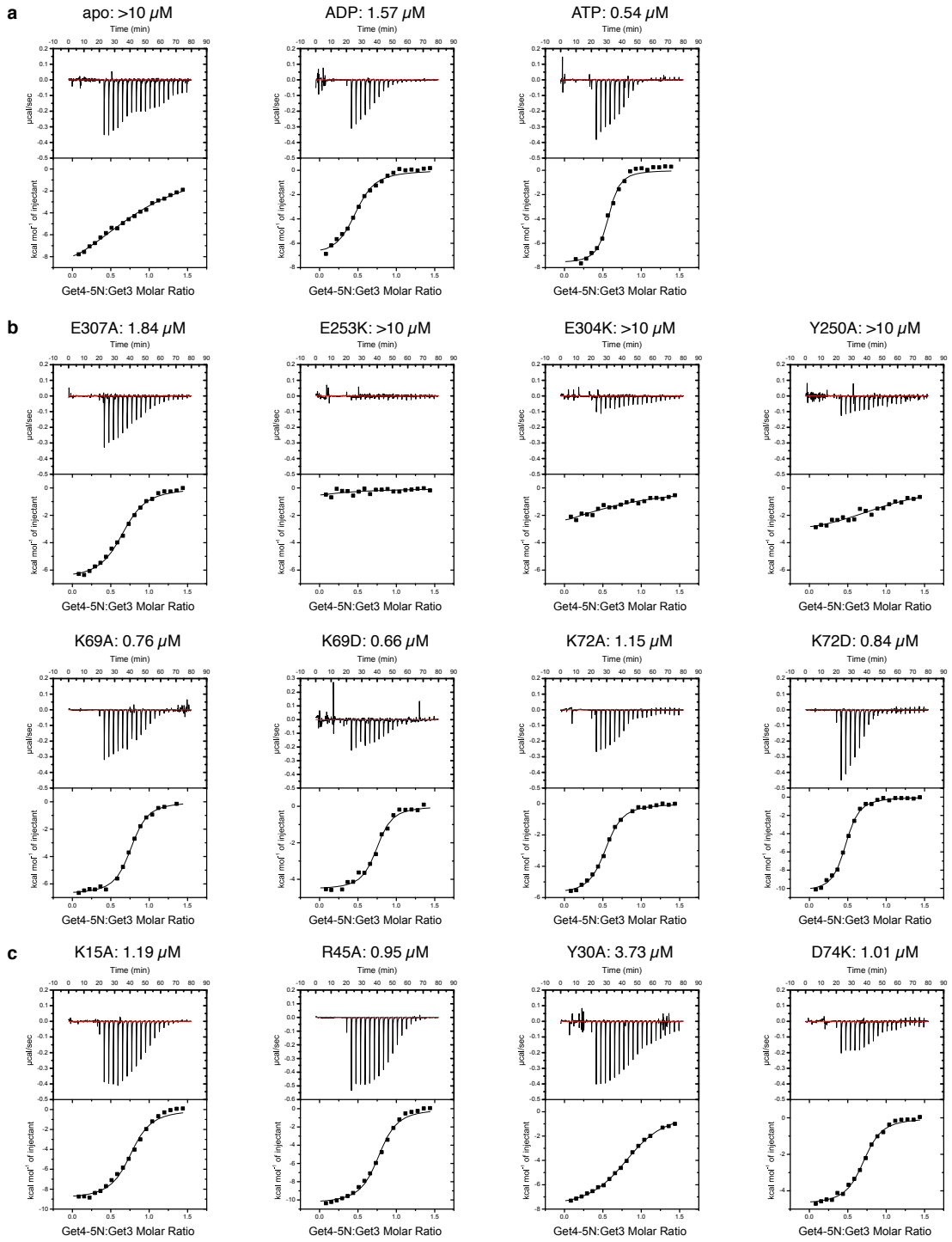
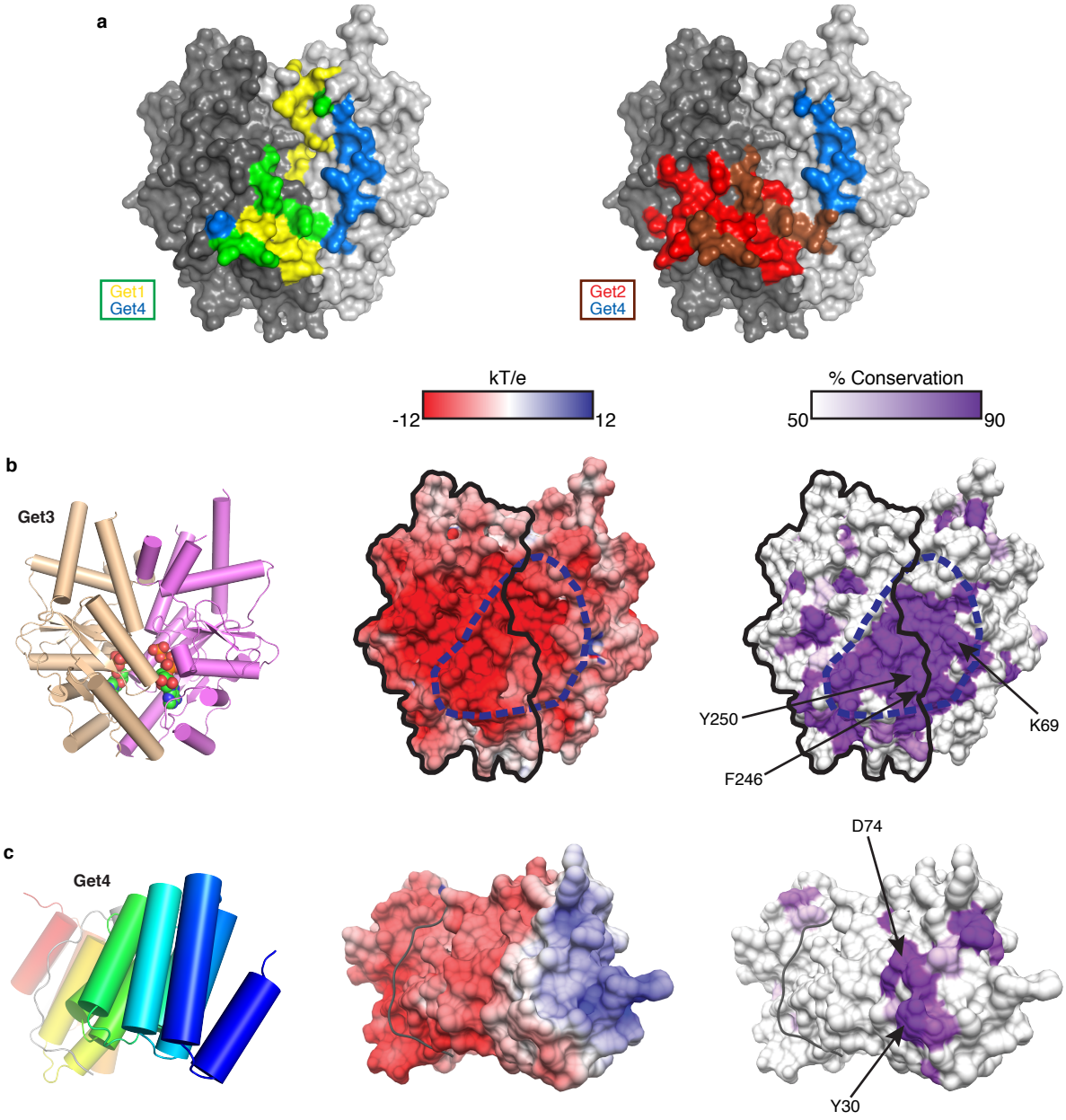
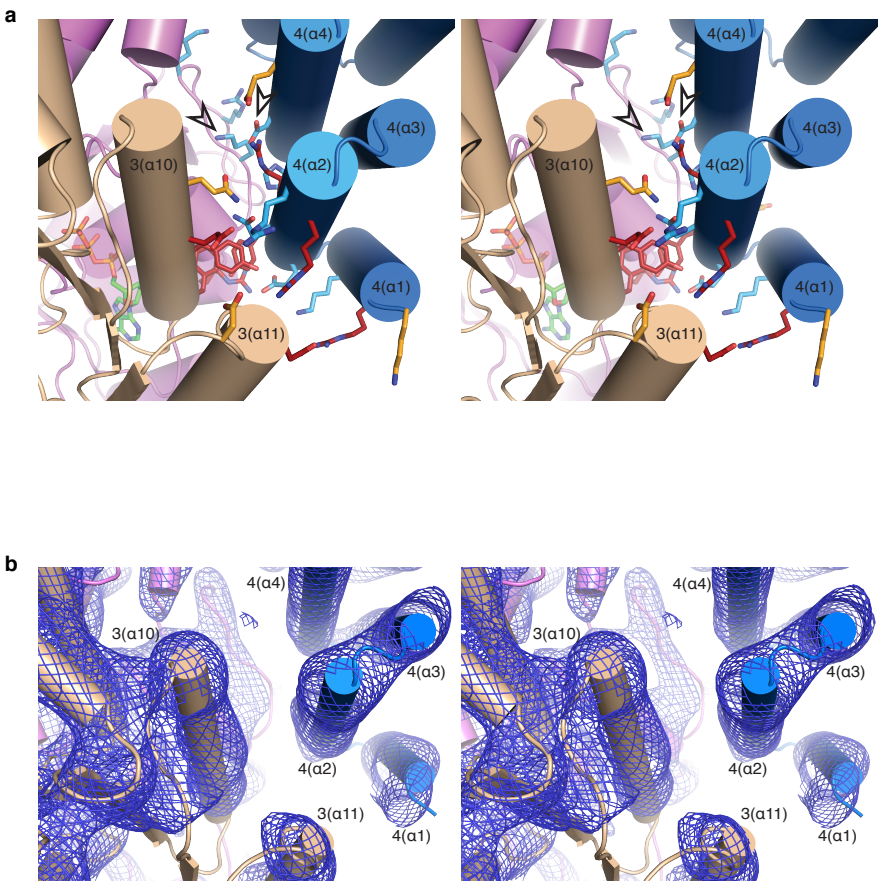


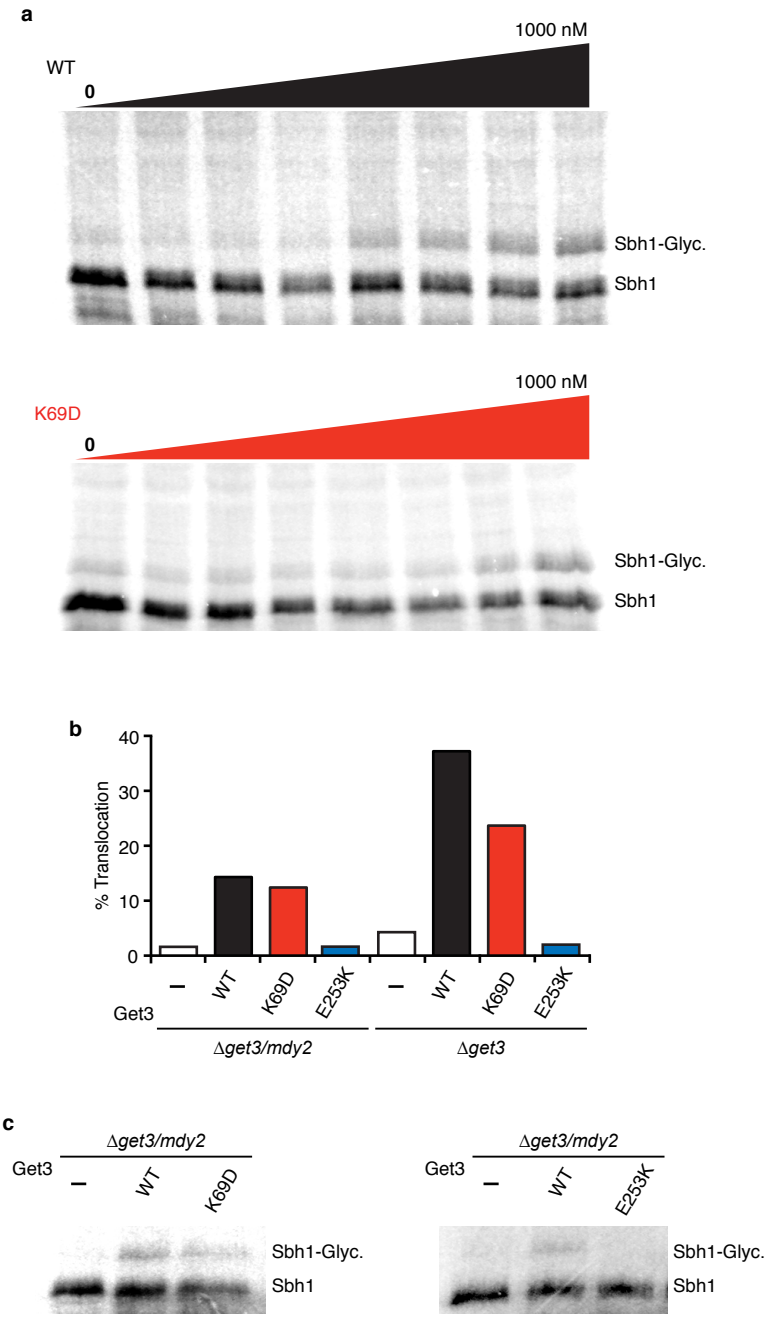
Supplementary Figure 1. Representative ITC isotherms for nucleotide dependent complex formation. (a) Representative measurements for Get3 in various nucleotide states. Raw data are shown in the top panel of each trial represented as the power input into the sample cell over time. Integrated data are shown in the bottom panels in terms of the total energy required for equilibration as a function of the molar ratio of Get4-5N:Get3. The solid line represents the best-fit model used to calculate affinities. The affinities indicated for each nucleotide state are the average from at least three experiments. In all experiments Get4-5N was titrated into the sample cell containing Get3. (b) Representative measurements for Get3 mutants from the Anchoring interface, top, or the Regulatory interface, bottom. The indicated affinity for each mutant represents the value obtained from a single experiment carried out in the presence of ATP. (c) Representative measurements for Get4 mutants from the various interfaces as in (b).



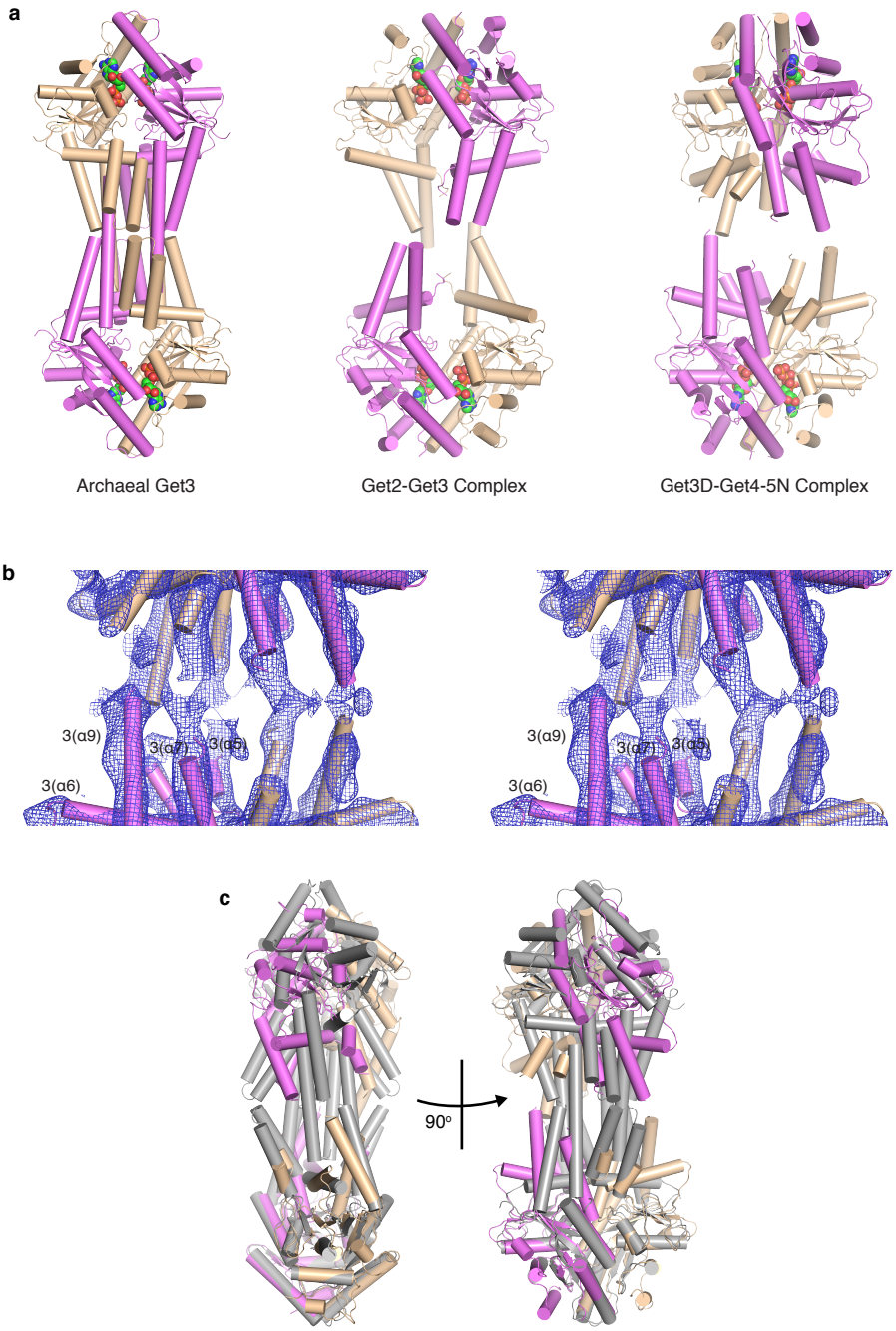
Supplementary Figure 2. Surface properties of Get3 and Get4. (a) Left, Surface representation of closed Get3 dimer (gray) from (b) showing Get4 binding site (blue), Get1 cytoplasmic domain binding site (yellow) and the overlap (green). Right, Similar surface representation highlighting the Get4 binding site (blue), Get2 cytoplasmic domain binding site (red) and the overlap (brown). (b) Left, orientation of Get3 used in subsequent panels to highlight the binding interfaces. Monomers are colored as in Figure 2. For surface representations one monomer is outlined in black and dashed lines (blue) highlight the interaction surface of Get4 on Get3. Center, accessible surface color ramped based on electrostatic potential from -12 kT/e (red) to +12 kT/e (blue). Get5N colored gray. Right, accessible surface color ramped based on conservation from ≤50% (white) to ≥90% (purple). Get5N colored gray. Coloring is based on a ClustalW [34] alignment using the following sequences: Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus fumigatus, Candida albicans, Pichia pastoris, Nematostella vectensis, Caenorhabditis elegans, Drosophila melanogaster, Danio rerio, Xenopus laevis, Mus musculus, Homo sapiens, Arabidopsis thaliana, Neurospora crassa, Anolis carolinensis. (c) Surface properties of Get4 as in (b), except Get4 is color-ramped from N-terminus (blue) to C-terminus (red).



Supplementary Figure 3. Stereo views of the Get3D-Get4-5N interface. (a) The Get3D-Get4 interface. Coloring is the same as in Figure 3a. Get3 Lys69 and Get4 Asp74 are highlighted by open arrowheads. (b) 2Fo-Fc Electron density contoured at 1.5σ highlighting the Get3D-Get4 interface.



Supplementary Figure 4. TA targeting assay. (a) Representative autoradiograph images used to calculate Get3 translocation efficiency corresponding to Fig. 4b for wildtype (Top) and K69D (Bottom). (b) Comparison of Get3 translocation efficiencies in Δ*get3* and Δ*get3/Δmdy2* lysates. (c) Representative autoradiograph images used to calculate Get3 translocation efficiencies for *Δget3/Δmdy2* lysate in (b).



Supplementary Figure 5. Tetramers of Get3. (a) Tetramers of Get3 seen in various crystal forms with Get3 dimers similar to Figure 5a,b. Each is highlighted with the archaeal Get3 (Methanocaldococcus jannaschii) from PDB ID 3UG6, the Get2-Get3 complex from PDB ID 3SJD, and the Get3D-Get4-5N crystal structure. (b) Stereo view of the 2Fo-Fc density contoured at 1.5σ showing the loops involved in formation of the Get3D-Get4-5N complex tetramer. (c) Comparison of the Archaeal MjGet3 dimer aligned to one dimer of the Get3D-Get4-5N complex dimer.

Supplementary Table 1. Summary of ATPase Data

Get3 ± Get4/5	k_{cat} (min^{-1}) Mean	SD[1]	*n*
WT	0.870	0.254	7
+ Get4/5	0.139	0.043	
E253K	0.566	0.034	2
+ Get4/5	0.536	0.030	
E304K	0.718	0.020	2
+ Get4/5	0.739	0.041	
K69A	0.847		1
+ Get4/5	0.132		
K69D	1.213	0.298	4
+ Get4/5	0.508	0.145	
K72A	1.417		1
+ Get4/5	0.203		
K72D	1.519	0.078	2
+ Get4/5	0.314	0.029	
WT	0.676	0.065	2
+D74K	0.462	0.107	
K69D	1.213	0.298	4
+D74K	0.135	0.004	2

[1]SD = standard deviation; *n* = number of trials

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